# MICROBIOTA-MEDIATED COMPETITION BETWEEN PEST INSECTS AND ITS APPLICATION TO THE EVOLUTION-PROOF PROTECTION OF CROPS

Antoine Rombaut[1], Romain Gallet[1], Kenza Qitout[1], Mukherjy Samy[1], Robin Guilhot[1], Pauline Ghirardini[1], Paul G. Becher[2], Anne Xuéreb[1], Patricia Gibert[3] and Simon Fellous[1*]

1 CBGP, INRAE, CIRAD, IRD, Montpellier SupAgro, Univ Montpellier, Montpellier, France

2 PGB Dept Plant Protection Biology - Chemical Ecology Horticulture, Swedish University of Agricultural Sciences, Alnarp, Sweden

3 Laboratoire de Biométrie et Biologie Evolutive UMR 5558, CNRS, Université Lyon 1, Université de Lyon, Villeurbanne, France

* author for correspondence and lead contact: Simon Fellous

simon.fellous@inrae.fr

**ORCIDs:**

Antoine Rombaut: 0000-0002-8994-351X

Romain Gallet: 0000-0002-7419-7403

Robin Guilhot: 0000-0003-1441-8486

Paul G. Becher: 0000-0001-5058-2668

Patricia Gibert: 0000-0002-9461-6820

Simon Fellous: 0000-0003-3921-4578

**Keywords:** microbiota ; symbiosis ; competition ; agroecology

**Author contributions:**

S.F., P.G. and A. R. designed research ; A.R., R. Ga. K. Q., M. S., R. Gu., P. G. and A. X. performed research; A. R., P.B., P. G. and S. F. analysed data ; S.F. wrote the paper with contributions from A. R., R. Gu., P.B. and P. G.

The PDF file includes:

- Main text
- Figures 1 to 5
- Table 1
- Supplementary materials 1 to 4

## Abstract

Species that share the same resources often avoid costly competition with context-dependent behaviors. This is the case of the invasive insect pest *Drosophila suzukii* which larvae's ecological niche overlaps with that of *Drosophila melanogaster* in ripe, but not rotten, fruit. Previous research showed *D. suzukii* prevents costly larval competition by not ovipositing in substrates with *D. melanogaster* eggs. We discovered *D. suzukii* females respond to cues produced by *D. melanogaster* adults associated to gut microbiota bacteria. This behavior of *D. suzukii* varied over time and among populations, revealing subtle condition-dependence. In particular, *D. suzukii* females that bore *D. melanogaster* gut bacteria stopped avoiding sites with *D. melanogaster* cues. The adaptive significance of the behavior was investigated by reproducing experimentally in-fruit larval competition. *D. suzukii* larvae only suffered from competition with *D. melanogaster* if the competitor was associated to its microbiota, suggesting *D. suzukii* has evolved a solution to avoid its offspring develops in challenging environments. We argue that *D. suzukii*'s competition avoidance behavior has features enabling the design of an evolution-proof repellent to protect crops.

## Introduction

Over the last 10 years, the Asian fly *Drosophila suzukii* (*Ds*) has spread in Europe and the
65 Americas (1) causing important fruit-production losses (2-4). As a consequence, research on this species has multiplied in pest-control and *Drosophila* laboratories. Numerous colleagues readily noticed the contamination of *Ds* cages with *D. melanogaster* (*Dmel*) individuals led to its rapid competitive exclusion (5). This phenomenon can be partly explained by the observation that *Ds* females avoid laying eggs in resource sites that already contain *Dmel*
70 eggs (6). *Ds* females however do not avoid oviposition on sites with conspecifics (6)(Fig. S1), prevention of larval crowding does therefore not explain female oviposition behaviour. We investigated the variability and mechanisms of *Ds* ovipositional response to *Dmel*-associated stimuli. The oviposition deterrent deposited by *Dmel* – a form of repellent *sensus lato* (7) – appeared to rely on symbiotic bacteria and be an adaptive response to microbiota-mediated
75 larval competition between the two species.

## Results and discussion

### *Variable response of* D. suzukii *females* to D. melanogaster *cues*

80 In an early experiment, we followed over four days the egg-laying preferences of groups of *Ds* females that were given the choice to oviposit either on control substrates or substrates previously exposed for 24h to *Dmel* females (Fig. 1). Oviposition substrates were replaced daily. During the first two days, *Ds* females laid the majority of their eggs on sites that had not been exposed to *Dmel*. However, *Ds* females did not show this avoidance during the final

85 two days (Fig. 2a). The experiment shows that *Ds* avoidance behavior is plastic and depends on female experience or condition.

In order to determine how widespread *Ds* avoidance behavior is among fly populations, we tested females from different laboratory populations founded with insects captured in 90 France (i.e. our reference population used throughout this study), the USA, China and Japan (see methods). Because in these behavioral investigations individual females were the essential unit of replication, and so as to consider potential inter-individual differences, this and all following experiments were carried out single females rather than groups of flies. Females from most populations exhibited avoidance but not those from the Japanese 95 population (Fig. 2b). *Ds* originates from mainland China, invaded Japan at the beginning of the 20$^{th}$ century and recently Europe and North-America (8). These results hence show that avoidance behavior is neither restricted to invasive populations nor to those from the area of origin.

100 We further meant to assess whether avoidance behavior was shared between laboratory and wild *Ds* flies. We therefore trapped wild *Ds* adults from the Montpellier region, Southern France, using classical vinegar traps modified to prevent the drowning of captured flies (see methods). These traps attracted various species of Drosophilid flies, among which a number of *Ds* females. Unlike laboratory *Ds* flies, wild *Ds* females did not exhibit avoidance behavior 105 to *Dmel*-exposed substrates (Fig. 2c). We identified three alternative explanations: (1) avoidance behavior is a laboratory artefact ; (2) uncontrolled fly age or pre-capture history affects female selectivity ; or (3) exposure to other Drosophilid flies, including *Dmel*, during

the time spent in traps suffices to trigger avoidance loss as we also observed in our first experiment (Fig. 2a).

110

Our results show that *Ds* oviposition avoidance of sites with *Dmel* cues varies among populations and with individual experience or physiological condition. This contrasts with the sustained and hard-wired oviposition avoidance that *Dmel* females display in response to geosmin (9), a molecule produced by microorganisms responsible for late-stage fruit rot
115 that are detrimental to *Dmel* larvae. Why and how *Ds* females lose response to *Dmel* cues (Fig. 2a) could be due to, at least, three non-excluding phenomena. First, Drosophila flies have remarkable learning abilities (10) ; *Ds* may therefore learn to tolerate these cues or habituate to them leading to oviposition avoidance loss. Second, females may lose oviposition site selectivity (i.e. take more risks) after 2 days of successful egg-laying. This
120 scenario is unlikely as the *Ds* females used in our experiments had had the opportunity to oviposit before experiment onset. Third, *Ds* exposure to *Dmel* or *Dmel* products (e.g. feces) could modify *Ds* internal state in such way that oviposition avoidance is altered because it is not possible or necessary any more.

125

**Conditions of repellency by *D. melanogaster***

The variable behavioral response of *Ds* prompted us to investigate whether *Dmel*-induced repellency was variable too. As published results and our initial experiments (Fig. 2a and Fig.
130 S1) had been carried out with *Dmel* females or eggs, we next compared the ability of *Dmel* males and females to induce repellency in more detail. The experiment showed that males,

like females, induced repellency (Fig. 3a). This rules-out *Dmel* eggs or oviposition associated cues as driving factors, and contrasts with Tephritid fruit-flies that avoid larval crowding using so-called host-marking pheromones (11).

135

We further investigated possible variations among *Dmel* populations and its sister species. *D. simulans* (*Dsim*), which ecology is very close to that of *Dmel* (12). We tested the repellency of wild *Dmel* trap-captured in Southern France, lab-reared F1 offspring of the same wild Southern France *Dmel* population, the Oregon-R lab strain of *Dmel* used for all previous
140 experiments. Similar to laboratory flies, wild *Dmel* flies were repellent, however those that had spent one generation in the laboratory were not (Fig. 3b). The *Dsim* population we tested was not repellent. Similarly, fruit exposure to *Ds* did not elicit *Ds* oviposition avoidance (Fig. S1). Repellency is therefore a feature of wild and laboratory *D. melanogaster* populations that may nonetheless be sensitive to rearing conditions.

145

Some of the above results suggest a plausible mechanism behind *Ds* oviposition avoidance and its plasticity. We observed a decrease of *Ds* avoidance behavior after 2 days of exposure to *Dmel* associated products (Fig. 2a) and that both males and females of *Dmel* produced the repellent (Fig. 3a). Besides, we carried-out an additional experiment testing whether
150 repellency restricted to *Dmel*-exposed substrates, or also protected surrounding sites. Lack of effect on substrates neighboring *Dmel*-exposed medium (Fig. S2) was reminiscent of the local effect of *Dmel* gut bacteria inoculated to nutritive media on *Dmel* adult foraging preferences (13, 14). It was shown indeed *Drosophila* foraging preferences depend on the combination of the bacterial species inoculated to the nutritive medium and that
155 symbiotically present in the fly (13). Drosophilids hence possess the sensory and neuronal

circuitry to perceive specific bacteria (or cues associated with them) and respond to their presence in function of their own symbionts. It was plausible to suspect a similar ability in *Ds* and hypothesized that microbial symbionts of *Dmel* can be perceived by *Ds* females, and that oviposition avoidance, or its lack, would be a function of *Ds* symbiotic status. We
160 describe below a series of experiment testing this hypothesis.

**Bacterial symbionts of *Dmel* are involved in repellency and *Ds* avoidance loss**

165 In order to investigate the implication of extra-cellular microorganisms in *Dmel* repellency, we tested whether axenic *Dmel* flies were repellent as conventional flies. Because we suspected *Ds* symbiotic status being involved in the loss of oviposition avoidance, we also compared the response of axenic and conventional *Ds* females. Axenic *Dmel* flies did not elicit significant oviposition avoidance in *Ds* (Fig. 4a). Extra-cellular micro-organisms thus
170 participate to *Dmel* repellency. However, both axenic and conventional *Ds* responded to the repellency provoked by conventional *Dmel* (Fig. 4a). It rules out extra-cellular micro-organisms as a necessary condition to perceive repellent cues.

We then tried to restore experimentally repellence in *Dmel* by inoculating axenic flies with
175 candidate bacteria (i.e. creating gnotobiotic flies). The bacterial microbiota of *Dmel* has been extensively described over the last ten years, showing it largely varies among populations and environmental conditions and often comprises species of the genera *Lactobacillus* and *Acetobacter* (15-18). We therefore elected to associate *Dmel* flies with a strain of *Lactobacillus brevis,* or with one of *Acetobacter pomorum*. Both these bacterial strains had

180 been isolated from a laboratory population of *Dmel* and are frequently used for microbiota studies (19, 20). In order to investigate whether any type of bacterium could restore repellency in axenic *Dmel,* we also associated *Dmel* flies with a strain of *Escherichia coli* previously shown as non-pathogenic to flies (21). *Dmel* inoculation with *L. brevis*, enabled *Dmel* repellency while *A. pomorum* and *E. coli* did not (Fig. 4b). This experiment confirms the
185 role of *Dmel* symbiotic association in repellency and shows its bacterial origin. It further implies all bacteria cannot elicit repellency, suggesting a form of specificity in the cues produced by *Dmel* and perceived by *Ds*. The bacterial microbiota of adult *Dmel* flies varies largely among populations and individuals (15-18). These variations likely explain the differences in terms of repellency intensity we observed in the different experiments we
190 conducted. For example, the absence of repellency by F1 offspring from wild *Dmel* may be due to the alteration of their microbiota during the first stages of domestication (22). However, the range of microorganisms able to render *Dmel* repellent cannot be determined on the basis of our study. Further work with wild microorganisms, flies and conditions will be necessary to assess the field prevalence of the symbiont-based phenomenon unveiled here.

195

Why did *Ds* females lose avoidance behaviour following exposure to *Dmel* cues (Fig. 2a)? We hypothesized the decrease of oviposition avoidance was due to colonisation of *Ds* by the microorganisms *Dmel* deposited on oviposition sites. In order to test this hypothesis, we mono-associated adult *Ds* for 5 days with the strain of *L. brevis* that elicited strong
200 repellence by *Dmel* (Fig 4b). As expected, *Ds* females associated with *L. brevis* did not avoid oviposition on substrate that had been exposed to *Dmel* adults bearing the same bacterium (Fig 4c). *Ds* females hence avoided sites with cues indicative of presence of *Dmel* unless they carried similar bacteria. Such plastic loss of avoidance behaviour would make sense, in

evolutionary terms, if the bacteria were detrimental to *Ds* larvae and that they were
205 transmitted vertically (or pseudo-vertically) from mothers to offspring (23-25). Contaminated mothers would not avoid poor quality oviposition sites as their offspring would anyways inherit their costly bacteria. Alternatively, avoidance loss could also be due to host manipulation by gut bacteria (26): as *Drosophila* larvae favour the development of the microorganisms they share fruit flesh with (27), a bacterium in *Ds* mothers could
210 manipulate its host in order to increase larval presence in sites where it is detected. Avoidance loss by bacterium-associated *Ds* females probably explains why trap-captured wild *Ds* females did not avoid *Dmel* cues (Fig. 2c). In the traps, wild *Ds* were in close contact with other Drosophilids from which they likely acquired a diversity of microorganisms.

215 To investigate the possibility of transferring our results to application in pest management, we investigated whether bacteria deposited by *Dmel* were sufficient to repel *Ds* oviposition even in absence of *Dmel* individuals, or if *Ds* flies perceive cues produced by the interaction between *Dmel* and its symbionts. To this end we carried out two experiments. In the first, we tested the repellence of medium inoculated with 1,000,000 bacterial cells. In the second,
220 we inoculated the medium with only 5,000 cells corresponding to the numeration of live bacteria on substrates exposed to *Dmel* in the conditions of our experiments. *Ds* females avoided oviposition on media inoculated with numerous bacterial cells. However, lower numbers of live bacterial missed to elicit avoidance behaviour (Fig. 4d), ruling out bacterial growth on medium as the sole source of repellence. Together, these two experiments
225 suggest that when *Dmel* adults are associated with bacteria, some compounds are produced, shed and later perceived by *Ds* females. A recent study reported another type of interaction between bacteria and their host that can affect egg-laying behavior (28). Bacteria deposited

during oviposition by the oriental fruit-fly, *Bactrocera dorsalis*, induce the synthesis by host fruits of a molecule, b-caryophyllene, that is perceived by females flies and subsequently
230 repels their oviposition. In the case of *Ds* ovipositional response, prospects for crop protection will necessitate identifying the compounds produced by the interaction between *Dmel* and its bacteria and testing them as pure molecules.

235 **_Ds_ larvae suffer from competition with symbiont-associated _Dmel_ larvae**

Avoidance behavior by *Ds* females could be an adaptation meant to ensure offspring do not develop in poor quality sites. In order to test whether *Ds* larvae suffer from competition with *Dmel* larvae, we reproduced in-fruit competition between the two species. Surface-sterilized
240 grape berries were pierced with a fine-needle and single *Ds* eggs deposited in each hole like *Ds* females do (Fig. S3a). Some holes also received axenic or conventional *Dmel* eggs. We deposited either 1 or 5 *Dmel* eggs per *Ds* egg. Number of eggs of each species were based on infestation intensities observed in field-collected fruit from which both species had emerged (29). *Ds* developmental success (i.e. proportion of eggs that reached adulthood) was
245 impaired by competition with *Dmel* larvae that were associated with their microbiota, but not with axenic *Dmel* larvae (Fig. 5). In the wild, *Dmel* eggs are never axenic, the normal outcome of larval competition is therefore poor *Ds* development. Results hence support our hypothesis that *Ds* oviposition behavior prevents costly larval competition with *Dmel*. It is noteworthy that unlike in (30), the presence of *Dmel* bacteria (in absence of *Dmel* larvae) -
250 which we obtained by exposing fruit to male *Dmel* - was not detrimental to *Ds* larval

development. This illustrates the environmental dependence of bacterial effects onto *Drosophila* phenotype (e.g. 31).

It Is remarkable that axenic *Dmel* larvae failed to reduce *Ds* larval development and probably
255 explains why *Ds* females did not respond to cues produced by axenic *Dmel*. *Ds* females would only avoid oviposition in microbial contexts that are detrimental to their offspring. Bacterial microbiota composition, and its effects on host phenotype, vary widely among *Drosophila* species, populations and individuals (18, 30). The plastic decision by Ds to oviposit, or not, in function of microbiota composition may therefore enable using all
260 suitable oviposition sites, and only avoid sites contaminated with costly competitors when necessary.

265 **Ecological significance and prospects for evolution-proof means of crop protection**

Our study shows the competition between insect species with overlapping ecological niches at the larval stage can be determined by microbial symbionts. Symbionts are further used by egg-laying *Ds* females to choose adequate oviposition sites so as to alleviate competition
270 costs. It is well established that microorganisms impact the outcome of competitive interactions between hosts (32). Often, parasitic microorganisms shed by tolerant species have detrimental effects on less-tolerant competitors (e.g. 33); the spill-over hypothesis that facilitates the spread of some invasive species is based on this very mechanism (32). Symbiotic microorganisms can also elicit beneficial effects for heterospecific neighbors;

275 mycorrhizal fungi can for example mediate mutualism between plants species (34). In the present case, bacteria beneficial to *Dmel* host appeared detrimental to *Ds* larvae, in complete opposition with how bacteria and yeast associated to *Ds* larvae facilitate fruit use by *Dmel* (29, 35). We hence document an original example of harmful interactions between competing insects mediated by their microbiota. Humans may exploit these interactions to
280 protect the food they grow.

*Ds* is responsible for heavy crop losses throughout the globe due to the development of larvae in farmed fruit. It is tempting to exploit *Ds* oviposition avoidance to shelter fruit from *Ds* damages. However, avoidance loss as described above (Fig. 2a, 4c) means *Dmel*'s bacteria
285 could not be sprayed directly in orchards, even more if the chosen microorganism is frequent in local *Ds* flies (Fig. 2b). A better solution may be to use as repellent the compounds produced by bacteria-inoculated *Dmel* (Fig. 4d). Obviously, it is assuming avoidance loss is based on *Ds* female colonization by repellent bacteria rather than habituation to the stimulus (10, 36). Otherwise this repellent could only be used once per fly
290 generation, which may be a problem as *Ds* lives several months in the field (37). Identifying *D. suzukii*'s chemosensory receptors and circuitry involved in the recognition of *Dmel* cues and its consequential behavioral response may also enable the design of an optimized repellent.

295 Society requests agricultural innovations to be sustainable. Resistance evolution has eroded the efficacy of numerous crop protection solutions. Environmentally-safe and evolution-proof alternatives, which do not lose their field efficacy over time, are therefore needed. This is attainable with solutions based on phenomena similar to the repellent against *Ds* we

studied here. In the present case, *Ds* females genetically resistant to the repellent – not to be mistaken with females that plastically lose avoidance after exposure to *Dmel* products - would deposit their eggs on fruit that bear the cues of *Dmel* microbiota. This would happen in farm treated hosts as in wild hosts. In Europe, *Ds* host range is very large, females oviposit and larvae develop in about a third of the wild fruit species tested (38). In treated fruits, resistant flies would have a fitness advantage, but in untreated wild hosts, resistant females would oviposit indistinctively in both pristine fruit and fruit infested with *Dmel*. The fitness of resistant alleles would therefore be diminished by the ecological cost of larval competition, a cost not paid by susceptible alleles. The fitness of resistant genotypes relative to susceptible ones, and therefore the likelihood of resistance spread, will depend on the relative frequency of *Ds-Dmel* co-infestation in the wild and their effect on larval performance in a diversity of conditions, the relative importance of wild and farmed hosts for *Ds* life-cycle and the efficiency of the repellent, which varied among experiments and could be over-ridden by the strong attraction highly-preferred crops induce. It is however unlikely *Ds* larvae evolve and gain superior competitive ability against *Dmel* as these two species have coexisted for thousands of years (see Text S4 Supplementary Discussion). The key feature of an evolution-proof solution based on our reasoning is that costs of resistance originate from ecological phenomena, long-established and frequent in non-treated sites. This will likely be encountered in other systems and species of agricultural and health importance. Providing sustainable management solutions probably is one of the greatest innovative challenges evolutionary biology may tackle in order to benefit society.

## Conclusions

We discovered females of the major agricultural pest *Ds* preferred to lay their eggs away
325 from sites bearing *Dmel* cues. They recognized molecules produced by the interaction of adult *Dmel* of both sexes with bacteria of their gut microbiota. *Ds* avoidance behavior revealed plastic as *Ds* inoculated with repellent bacteria stopped avoiding the cues produced by *Dmel* flies associated with the same microorganism. Avoidance behavior appeared as an adaptation by *Ds* to avoid larval competition against *Dmel* larvae, which revealed deleterious
330 only when associated to their extra-cellular symbionts. We argue crop protection solutions based on phenomena featuring ecological costs of resistance, as we unveiled here, could reveal evolution-proof, not giving rise to resistance emergence.

## 335 Materials and Methods

### *General experimental design*

The study is based on a simple assay where female *D. suzukii* (*Ds*) are given the choice to lay
340 eggs on two substrates: either a blank control or a substrate that had previously been exposed to *D. melanogaster* (*Dmel*) adults (Fig. 1). By changing the nature of the *Ds* and *Dm* flies employed, we were able to reveal the factors that govern *Dmel*'s repellence and *Ds*'s corresponding avoidance.

In most cases, a single *Ds* female was placed in a 9cm diameter plastic cylindrical box for
345 24h. Boxes contained two 2*2cm*2cm plastic receptacles each half-filled with oviposition substrates, generally an agar-jellified strawberry puree or a piece of strawberry inserted in

blank agar. These two substrates were prepared the day before, one of them was exposed to 3 adult *Dmel* flies overnight. Because these experiments were conducted over 5 years with variable objectives, some experimental details varied among assays. In all experiments, a variable fraction of assayed females (usually around 50%) did not oviposit during the 24h they spent with the tested substrates ; these females were excluded from further analyses. Table 1 describes the experimental details, sample sizes and statistical analyses of each of the results reported in the article.

All flies were reared, and experiments conducted, in climatic chamber with a 13h:23°C/11h:19°C day/night cycle, an artificial dawn and dusk of 45min. Humidity was maintained constant at 75% relative humidity.

***Biological material***

Most experiments were carried out with our standard *Ds* population that was founded in 2013 by a few dozen individuals that emerged from blackberries harvested in Gaujac, Southern France (44.0794, 4.5781) ; and the classical *Dmel* population Oregon R, founded in 1927 and shared among laboratories since then. These fly colonies were maintained in standard drosophila vials with banana artificial medium (see below) or 30 cm cubic cages when we needed larger numbers of flies.

Additional laboratory populations of *Ds* were as follow. The Japanese population was founded from individuals captured in Matsuyama, Japan (33.8389, 132.7917) in 2015, the US population in Watsonville, California, USA (36.9144, -121.7577) in 2014, and the Chinese population in Shiping, China (23.7048, 102.5004) in 2015. The *D. simulans* population tested

370 was founded from individuals captured in 2015 in Lyon, France (45.7835, 4.8791). All populations were initially composed of a few individuals and experienced repeated population bottlenecks during maintenance. They were thus largely inbred at the time of testing in 2017.

375 Wild *Ds* were captured during summer 2016 in two localities 10km apart near Montpellier, Southern France (43.6816, 3.8776), and tested about a week after capture, once they started laying eggs in the laboratory. Wild *Dmel* were also captured near Montpellier. For the experiment reported in Fig. 3b, *Dmel* flies were captured in several instances. Flies from a first group were reared in the laboratory and their offspring (*i.e.* F1) tested along with
380 freshly-captured flies (*i.e.* F0). All wild flies were captured using custom-designed traps based on c.300 mL plastic cups, covered with cling-film, pierced on the sides for fly entry and containing an attractant (a mix water, vinegar, wine and sugar) separated from the flies by netting. The netting prevented fly drowning but allowed occasional access to the attractant as cups were readily shaken by wind or operators, which provoked netting soaking with
385 liquids. Traps were checked daily and usually contained various fly species, including *Dmel* and *Ds*.

***Recipes for rearing and oviposition media***

390 Laboratory flies were reared on custom banana medium (1.2 L water, 280 g frozen organic banana, 74 g glucose, 74 g inactivated baker's yeast, 12 g agar, 6 g paraben in 30 mL ethanol). The Chinese *Ds* population was reared in carrot medium (1.2 L water, 45 g carrot

powder, 45 g glucose, 27 g inactivated baker's yeast, 18 g corn meal, 13.5 g agar, 6 g paraben in 30 mL ethanol and 4 mL propionic acid).

395 In most cases, oviposition was assayed on strawberry puree (200 g frozen strawberry, 400 mL water, 6 g agar, 37 g glucose, 4 g paraben in 15 mL ethanol). In several instances (Table 1), we used jellified grape juice (100 mL commercial grape juice, 100 mL water, 12 g glucose, 2 g agar). Oviposition was also tested on pieces of strawberry inserted in jellified water (100 mL water, 1 g agar), they were first bleached (0.6% bleach during 5 min) to remove

400 contaminants.

***Axenics, mono-associated flies and microbiological work***

Axenic flies were produced following a protocol derived from (39). Briefly, *Drosophila* eggs

405 were collected on grape-juice medium (see previous recipes section) before being bleached and rinsed twice (1.2% sodium hypochlorite). Eggs were then transferred to 50 mL centrifugation vials with 10 mL autoclaved banana medium (see recipes section) which lids were either incompletely screwed of harboured breathing membranes. All manipulations were conducted under a laminar flow hood. With care, it is possible to transfer freshly

410 emerged adults to new vials aseptically and therefore maintain the population microbe-free for several generations.

To produce mono-associated (i.e. gnotobiotic) adult flies, vials containing axenic flies were inoculated with suspensions (i.e. $>10^5$ cells) of the relevant bacterium at least 4 days before experiment onset. Adults used for an experiment were discarded to avoid contamination of

415 subsequent assays.

***Larval competition between D. suzukii and D. melanogaster in fruit***

This assay aimed at testing whether the development of *Ds* larvae was affected by the
420 presence of *Dmel* larvae and their associated microbiota. We took great care of reproducing field-like conditions (i.e. in-fruit interactions) as competition costs notoriously depend on ecological conditions (e.g. 40) and the effects of *Drosophila* bacterial symbionts on larval development change with medium richness (e.g. 41). A key parameter was to choose a fruit species in which both *Ds* and *Dmel* had been reported to develop simultaneously in the field,
425 we elected grape (29). Given the large effect of grape variety on Ds oviposition (42), we first ensured *Ds* accepted to oviposit on the batch of grapes we used (fruit of an unknown cultivar bought in April 2018 in a food retail store) and that this behaviour was reduced by exposure to *Dmel* (data not shown). In order to mimic realistic competition conditions we manually pierced the skin of grape berries with fine needles, making a hole close in size as
430 those *Ds* females do with their ovipositor (43). Each hole was first inoculated with a wild strain of the yeast *Hanseniaspora meyeri* isolated from wild *Ds* adults and received a single *Ds* egg (Fig. S3a). There were 6 holes per berry. Each of these holes also received 0, 1 or 5 *Dmel* eggs. The larger ratio of *Dmel* to *Ds* eggs was chosen as it reflects relative infestation intensity observed in grapes collected in the field (e.g. 29). In half the cases, deposited *Dmel*
435 eggs had been made axenic by bleaching (see previous section on the production of axenic flies). Note that an important design choice was to either compare the effect of axenic and conventional *Dmel* larvae, or axenic *Dmel* and axenic *Dmel* artificially inoculated with microbes harvested from conventional flies. We rejected the second option because it would have been impossible to been certain that eggs artificially associated to microorganisms
440 cocktails bore all relevant strains. By contrast, the differential mortality of bleached (i.e.

axenic) and non-bleached (i.e. conventional) eggs could be controlled for statistically (see statistical analysis section below ; Fig. S3b) In the treatments without *Dmel* eggs but with its microbiota, pierced berries were exposed to 10 *Dmel* males for 24h prior to *Ds* egg deposition. All grape berries were incubated in individual plastic vials until adult flies
445 emerged. This assay comprised 25-30 individual berries per treatment (50 replicates for the control treatment with *Ds* eggs and no *Dmel* microbiota) spread over 8 temporal blocks. For brevity, we only present here the results for *Ds* survival until emergence, other results – which are largely congruent with those in this manuscript – will be published separately.

450 ***Statistical analyses***

In all reported experiments, but the one on larval competition (Fig. 5), *Ds* females deposited their eggs on either treated or untreated oviposition substrates. Egg counts on each type of medium were therefore not independent as they were produced by the same females.
455 Besides, total number of eggs varied among females and experiments and largely followed a Poisson distribution, which prevented the use of traditional linear models that assume normal distributions of the residuals. We therefore used a simple, robust statistical approach to analysing the proportion of eggs deposited on treated and untreated site: a non-parametric, one-tailed Wilcoxon signed rank test that took into account data pairing, was
460 compatible with its distribution and is often used in comparable studies (e.g.13). We however noticed that paired t-tests, which assume data follows a normal distribution, provided similar results. Importantly, the aim of our experiments was to investigate female behaviour determinants rather than infestation intensities, the units of replication hence were the females and their individual preferences towards different types of substrates. For

465 this reason, the statistical methods we employed were not affected by female fecundity variations: in other words, the most fertile females could not skew the results towards their specific preferences. With this in mind, it appeared preferable to include all females that oviposited, even if a single egg. Because of the plasticity of the avoidance behaviour, all experiments included a positive control - usually the response of standard *Ds* to laboratory
470 *Dmel* flies. This ensured lack of avoidance in an experiment was not due to unidentified factors or inappropriate conditions. Note several of our most important results were repeatedly observed in distinct experiments ; compare, for example, loss of avoidance in Fig. 2a and Fig. 4c, effect of axenic *Dmel* in Fig. 4a and Fig. 4b, restauration of *Dmel* repellency by *Lactobacillus brevis* inoculation in Fig. 4b and Fig. 4c.

475

Results from the larval competition assay were analysed using a linear mixed-model with the REML method. Numbers of *Ds* adult that emerged from each fruit were Log(x+1)-transformed and complied with tests assumptions. This model contained discreet, fixed terms describing the number of *Dmel* eggs deposited, whether *Dmel* microbiota was present
480 and their interaction. It was also very important that the model included the (log-transformed) number of *Dmel* adults that emerged from the fruit as a fixed, continuous factor. Indeed, this term was necessary to control for the additional mortality bleaching provoked in *Dmel* larvae of the axenic treatment (Fig. S3b). The presence of this term in the analysis ensures the significant effect of axeny was not an artefact due to reduced *Dmel*
485 larvae numbers. The model also included a block term (treated as random). Differences among treatments were tested with independent contrasts and pairwise student's tests.

All analyses were carried out with the software JMP 14.0 (SAS Institute Inc. 2018). Throughout the manuscript, stars in figures indicate significance of one-tailed statistical tests: * $p<0.05$; ** $p<0.01$; ***$p<0.001$; n.s. $p>0.05$.

All data is available on the Zenodo platform under the reference: 10.5281/zenodo.3970737.

**Acknowledgements:** We are grateful to Marc Kenis and Pierre Girod for providing the Chinese *D. suzukii* population ; Marie-Pierre Chapuis and Laure Benoit for help with microbial work.

**Funding:** This work received financial support from French ANR's 'Investissements d'avenir' (ANR-10-LABX-0001-01), Labex Agro, CIVC, BIVB ; ANR SWING (ANR-16-CE02-0015) ; INRA's department 'Santé des Plantes et Environnement' ; Eranet LEAP Agri projet Pest Free Fruit (ANR-18-LEAP-0006-02).

505

## References


1. Fraimout A, Debat V, Fellous S, Hufbauer R, Foucaud J, Pudlo P, et al. Deciphering the routes of invasion of *Drosophila suzukii* by means of ABC random forest. Mol Biol Evol. 2017.

510 2. De Ros G, Conci S, Pantezzi T, Savini G. The economic impact of invasive pest Drosophila suzukii on berry production in the Province of Trento, Italy. Journal of Berry Research. 2015;5(2):89-96.

3. Yeh DA, Drummond FA, Gómez MI, Fan X. The Economic Impacts and Management of Spotted Wing Drosophila (Drosophila Suzukii): The Case of Wild Blueberries in Maine.

515 Journal of Economic Entomology. 2020;113(3):1262-9.

4. Mazzi D, Bravin E, Meraner M, Finger R, Kuske S. Economic Impact of the Introduction and Establishment of Drosophila suzukii on Sweet Cherry Production in Switzerland. Insects. 2017;8(1).

5. Dancau T, Stemberger TL, Clarke P, Gillespie DR. Can competition be superior to

520 parasitism for biological control? The case of spotted wing Drosophila (Drosophila suzukii), Drosophila melanogaster and Pachycrepoideus vindemmiae. Biocontrol Science and Technology. 2017;27(1):3-16.

6. Shaw B, Brain P, Wijnen H, Fountain MT. Reducing Drosophila suzukii emergence through inter-species competition. Pest management science. 2017.

525 7. Deletre E, Schatz B, Bourguet D, Chandre F, Williams L, Ratnadass A, et al. Prospects for repellent in pest control: current developments and future challenges. Chemoecology. 2016:1-16.

8. Asplen MK, Anfora G, Biondi A, Choi D-S, Chu D, Daane KM, et al. Invasion biology of spotted wing Drosophila (Drosophila suzukii): a global perspective and future priorities. J Pest Sci. 2015;88(3):469-94.

9. Stensmyr Marcus C, Dweck Hany KM, Farhan A, Ibba I, Strutz A, Mukunda L, et al. A Conserved Dedicated Olfactory Circuit for Detecting Harmful Microbes in Drosophila. Cell. 2012;151(6):1345-57.

10. Kahsai L, Zars T. Learning and memory in Drosophila: behavior, genetics, and neural systems. International review of neurobiology. 99: Elsevier; 2011. p. 139-67.

11. Silva MA, Bezerra-Silva GCD, Mastrangelo T. The host marking pheromone application on the management of fruit flies-a review. Brazilian Archives of Biology and Technology. 2012;55(6):835-42.

12. Capy P, Gibert P. Drosophila Melanogaster, Drosophila Simulans: so Similar yet so Different. Genetica. 2004;120(1):5-15.

13. Wong AC-N, Wang Q-P, Morimoto J, Senior AM, Lihoreau M, Neely GG, et al. Gut microbiota modifies olfactory-guided microbial preferences and foraging decisions in Drosophila. Curr Biol. 2017;27(15):2397-404. e4.

14. Qiao H, Keesey IW, Hansson BS, Knaden M. Gut microbiota affects development and olfactory behavior in Drosophila melanogaster. Journal of Experimental Biology. 2019;222(5).

15. Chandler JA, James PM, Jospin G, Lang JM. The bacterial communities of *Drosophila suzukii* collected from undamaged cherries. PeerJ. 2014;2:e474.

16. Staubach F, Baines JF, Künzel S, Bik EM, Petrov DA. Host species and environmental effects on bacterial communities associated with Drosophila in the laboratory and in the natural environment. PloS one. 2013;8(8):e70749.

17. Wang Y, Kapun M, Waidele L, Kuenzel S, Bergland AO, Staubach F. Common structuring principles of the Drosophila melanogaster microbiome on a continental scale and between host and substrate. Environmental Microbiology Reports. 2020;12(2):220-8.

555 18. Chandler JA, Morgan Lang J, Bhatnagar S, Eisen JA, Kopp A. Bacterial Communities of Diverse *Drosophila* Species: Ecological Context of a Host–Microbe Model System. PLoS Genet. 2011;7(9):e1002272.

19. Ryu J-H, Kim S-H, Lee H-Y, Bai JY, Nam Y-D, Bae J-W, et al. Innate Immune Homeostasis by the Homeobox Gene *Caudal* and Commensal-Gut Mutualism in *Drosophila*.
560 Science. 2008;319(5864):777.

20. Shin SC, Kim S-H, You H, Kim B, Kim AC, Lee K-A, et al. *Drosophila* Microbiome Modulates Host Developmental and Metabolic Homeostasis via Insulin Signaling. Science. 2011;334(6056):670.

21. Tracy C, Krämer H. Escherichia coli Infection of Drosophila. Bio-protocol. 2017;7(9).

565 22. Chen B, Du K, Sun C, Vimalanathan A, Liang X, Li Y, et al. Gut bacterial and fungal communities of the domesticated silkworm (Bombyx mori) and wild mulberry-feeding relatives. The ISME Journal. 2018;12(9):2252-62.

23. Pais IS, Valente RS, Sporniak M, Teixeira L. Drosophila melanogaster establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. PLoS Biol.
570 2018;16(7):e2005710.

24. Starmer WT, Peris F, Fontdevila A. The transmission of yeasts by Drosophila buzzatii during courtship and mating. Animal behaviour. 1988;36(6):1691-5.

25. Téfit MA, Gillet B, Joncour P, Hughes S, Leulier F. Stable association of a Drosophila-derived microbiota with its animal partner and the nutritional environment throughout a fly
575 population's life cycle. J Ins Physiol. 2018;106:2-12.

26. Johnson KVA, Foster KR. Why does the microbiome affect behaviour? Nat Rev Microb. 2018;16(10):647-55.

27. Stamps JA, Yang LH, Morales VM, Boundy-Mills KL. *Drosophila* Regulate Yeast Density and Increase Yeast Community Similarity in a Natural Substrate. PLoS ONE. 2012;7(7):e42238.

28. Li H, Ren L, Xie M, Gao Y, He M, Hassan B, et al. Egg-Surface Bacteria Are Indirectly Associated with Oviposition Aversion in Bactrocera dorsalis. Curr Biol. 2020.

29. Rombaut A, Guilhot R, Xuéreb A, Benoit L, Chapuis M-P, Gibert P, et al. Invasive *Drosophila suzukii* facilitates *Drosophila melanogaster* infestation and sour rot outbreaks in the vineyards. Royal Society Open Science. 2017.

30. Bing X, Gerlach J, Loeb G, Buchon N. Nutrient-Dependent Impact of Microbes on <em>Drosophila suzukii</em> Development. mBio. 2018;9(2).

31. Guilhot R, Rombaut A, Xuéreb A, Howell K, Fellous S. Environmental specificity in *Drosophila*-bacteria symbiosis affects host developmental plasticity. Evol Ecol. 2020;*In Press*(*In Press*).

32. Amsellem L, Brouat C, Duron O, Porter SS, Vilcinskas A, Facon B. Importance of microorganisms to macroorganisms invasions: is the essential invisible to the eye?(The Little Prince, A. de Saint-Exupéry, 1943). Advances in Ecological Research. 57: Elsevier; 2017. p. 99-146.

33. Tompkins D, White AR, Boots M. Ecological replacement of native red squirrels by invasive greys driven by disease. Ecol Lett. 2003;6(3):189-96.

34. Selosse M-A, Richard F, He X, Simard SW. Mycorrhizal networks: des liaisons dangereuses? Trends Ecol Evol. 2006;21(11):621-8.

35. Solomon GM, Dodangoda H, McCarthy-Walker T, Ntim-Gyakari R, Newell PD. The
600 microbiota of Drosophila suzukii influences the larval development of Drosophila melanogaster. PeerJ. 2019;7:e8097.

36. Little CM, Chapman TW, Hillier NK. Considerations for Insect Learning in Integrated Pest Management. Journal of Insect Science. 2019;19(4).

37. Panel A, Zeeman L, van der Sluis B, van Elk P, Pannebakker B, Wertheim B, et al.
605 Overwintered Drosophila suzukii Are the Main Source for Infestations of the First Fruit Crops of the Season. Insects. 2018;9(4):145.

38. Poyet M, Le Roux V, Gibert P, Meirland A, Prévost G, Eslin P, et al. The wide potential trophic niche of the Asiatic fruit fly Drosophila suzukii: the key of its invasion success in temperate Europe? PloS one. 2015;10(11):e0142785.

610 39. Koyle ML, Veloz M, Judd AM, Wong AC-N, Newell PD, Douglas AE, et al. Rearing the fruit fly Drosophila melanogaster under axenic and gnotobiotic conditions. JoVE (Journal of Visualized Experiments). 2016(113):e54219.

40. Kraaijeveld AR, Godfray HC. Trade-off between parasitoid resistance and larval competitive ability in *Drosophila melanogaster*. Nature. 1997;389(6648):278-80.

615 41. Storelli G, Defaye A, Erkosar B, Hols P, Royet J, Leulier F. Lactobacillus plantarum Promotes Drosophila Systemic Growth by Modulating Hormonal Signals through TOR-Dependent Nutrient Sensing. Cell Metabolism. 2011;14(3):403-14.

42. Mazzetto F, Lessio F, Giacosa S, Rolle L, Alma A. Relationships between Drosophila suzukii and grapevine in North-western Italy: seasonal presence and cultivar susceptibility.
620 Bulletin of Insectology. 2020;73(1):29-38.

43. Atallah J, Teixeira L, Salazar R, Zaragoza G, Kopp A. The making of a pest: the evolution of a fruit-penetrating ovipositor in *Drosophila suzukii* and related species.

Proceedings of the Royal Society of London B: Biological Sciences. 2014;281(1781):20132840.

625

## Figures and Tables

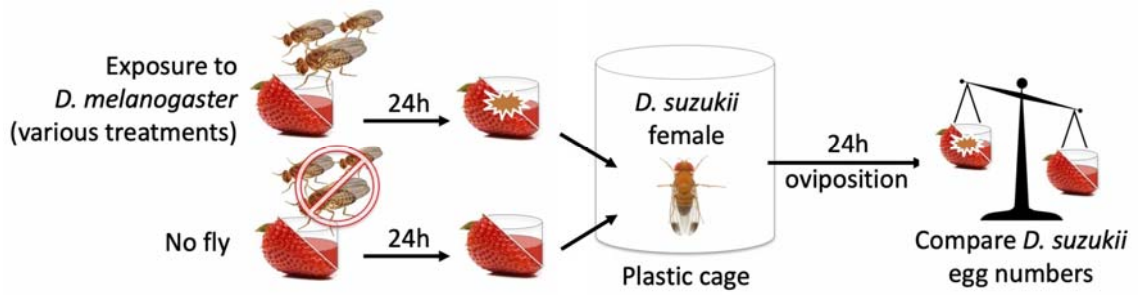



Figure 1: Schematic drawing of the experimental procedure for testing *D. suzukii* oviposition avoidance of egg-laying sites previously exposed to *D. melanogaster*. Details of each experiment, among which origin, sex and numbers of *D. suzukii* and *D. melanogaster* flies, cage size and oviposition substrate, are described in Table 1 of the Materials and Methods.

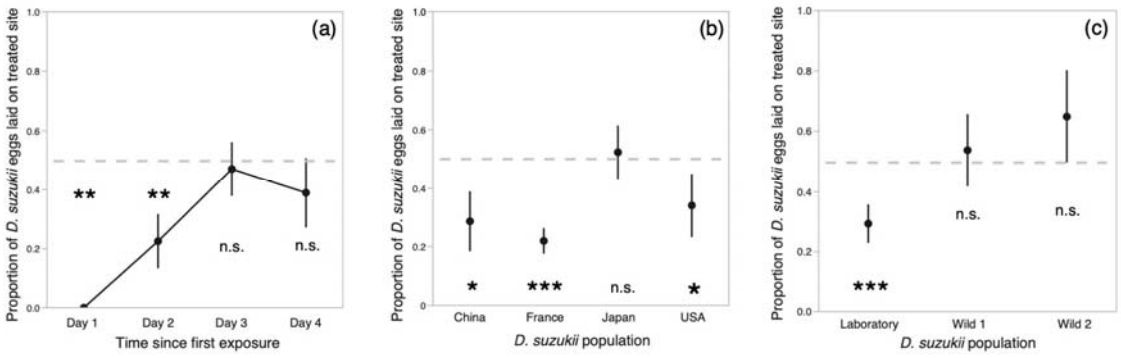



Figure 2: oviposition avoidance of *D. suzukii* females for egg-laying site previously exposed to *D. melanogaster*. Values significantly below 0.5 indicate *D. suzukii* preference for sites unexposed to *D. melanogaster*. Repeated tests of the same females (a) show plastic avoidance loss. *D. suzukii* populations from different geographical origins (b) exhibit variable avoidance. Trap-captured, wild *D. suzukii* females (c) did not avoid oviposition on *D. melanogaster* exposed substrates. Symbols indicate means and error-bars standard errors. Significant deviation from equal number of eggs on sites exposed to *D. melanogaster*, or control sites, were produced by one-tailed Wilcoxon signed rank tests ; * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$.

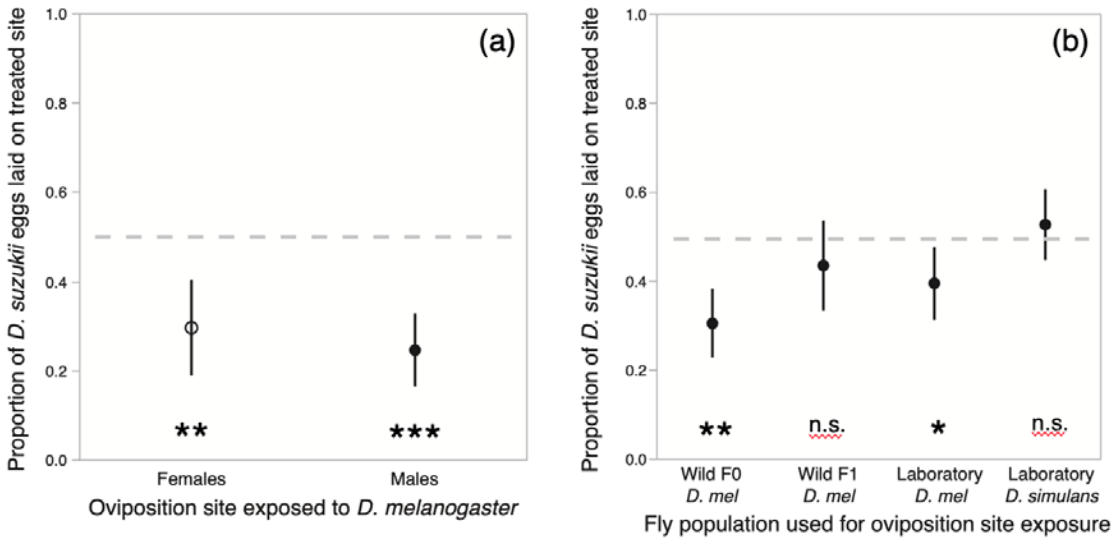



Figure 3: oviposition preferences of *D. suzukii* females for egg-laying site previously exposed to different *Drosophila* flies. *D. melanogaster* males, like females, (a) induce repellency. Trap-captured, wild *D. melanogaster* flies (F0 in b) induced repellency, however this property was not induced by laboratory-reared offspring from wild-caught flies (F1 in b) nor by *D. simulans* . Symbols indicate means and error-bars standard errors. Statistical tests produced by Wilcoxon signed rank tests; * for $p < 0.05$; ** for $p < 0.01$; *** for $p < 0.001$.

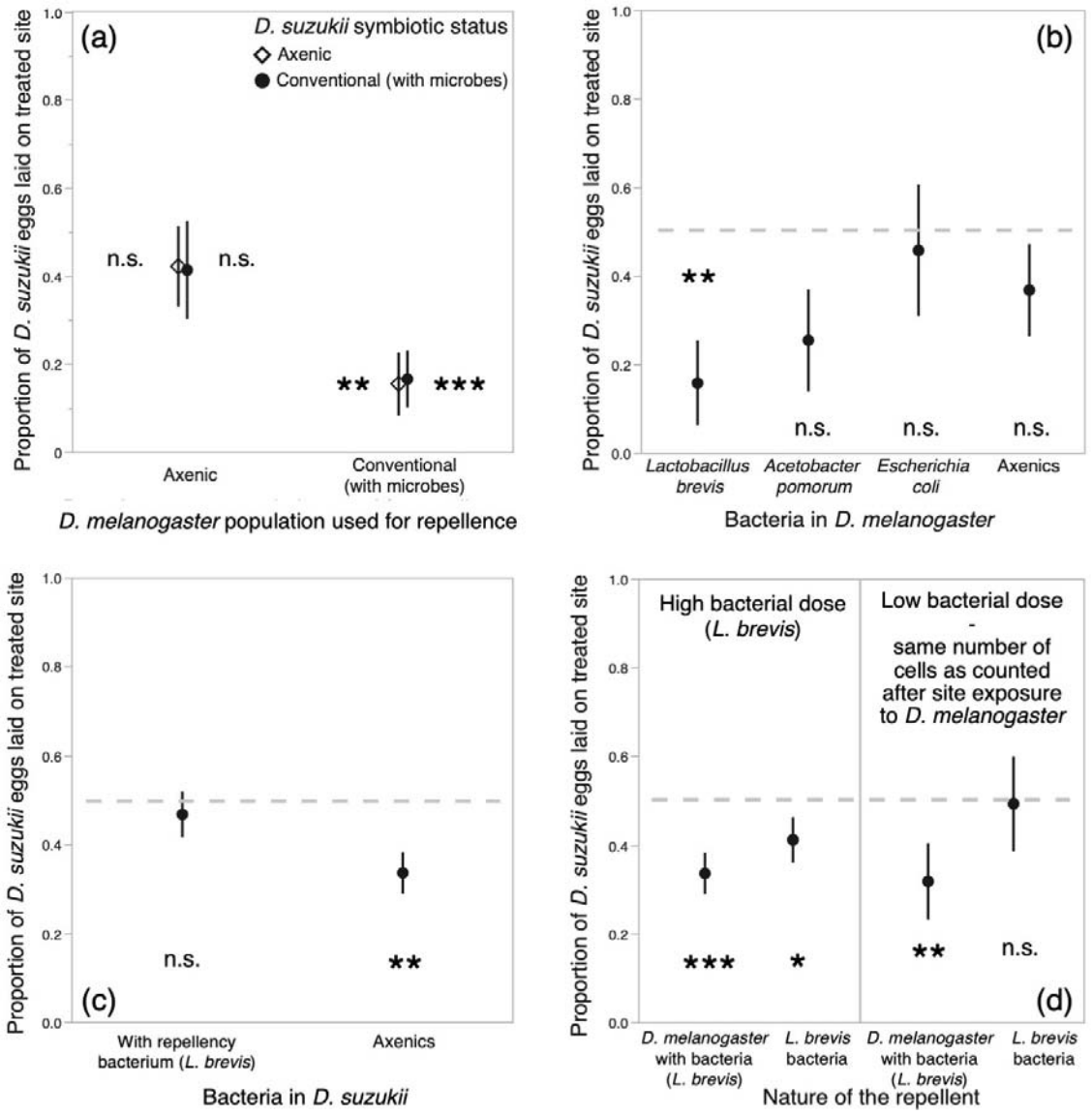
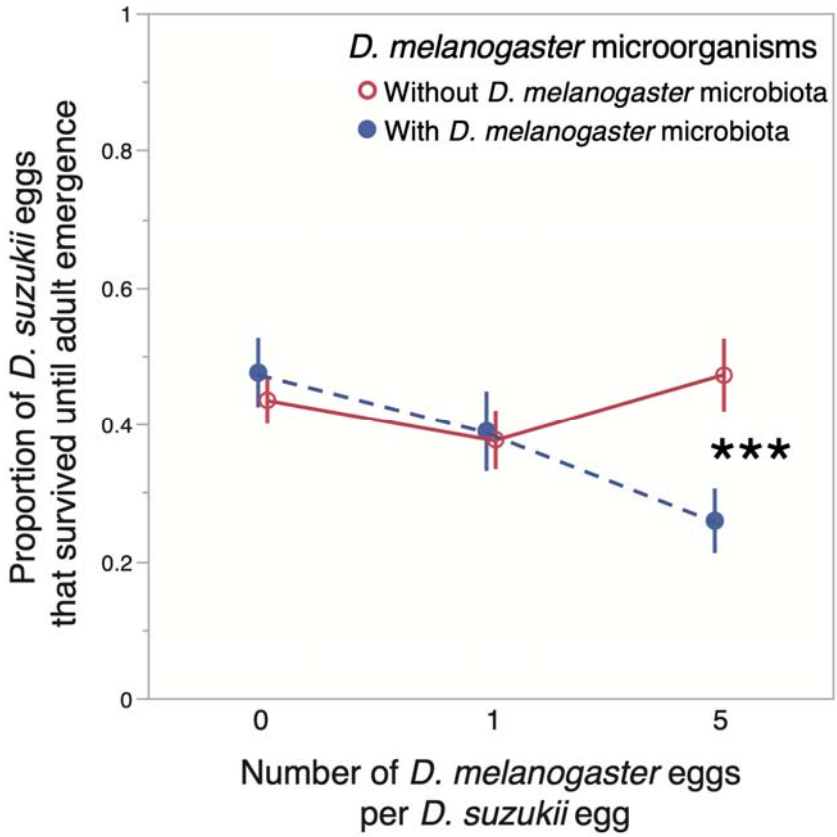



Figure 4 : investigation of the role of extra-cellular symbionts on *D. melanogaster* repellence and *D. suzukii* oviposition avoidance. Axeny, the removal of extra-cellular microorganisms, (a) had different effects on *D. melanogaster* and *D. suzukii*. Oviposition sites exposed to axenic *D. melanogaster* were
660 not avoided by *D. suzukii*, showing the implication of symbionts in *D. melanogaster* for repellence. By contrast, axenic *D. suzukii* behaved like conventional flies; *D. suzukii* microorganisms were therefore not mandatory for perceiving the repellent. Test of candidate bacteria in association with *D. melanogaster* (b) revealed the bacterium *Lactobacillus brevis* can restore repellence in formerly axenic flies (note the axenic and *Acetobacter pomorum* treatments were marginally non-significant,

665 p= 0.071 and p= 0.075, respectively). We hypothesized *D. suzukii* avoidance loss was due to their colonisation with *D. melanogaster* symbionts. As expected, *D. suzukii* females experimentally associated with the bacterium *L. brevis* (c) did not avoid oviposition on sites exposed to *L. brevis*-associated *D. melanogaster*. Direct inoculation of medium with *L. brevis* cells (d) in large numbers or at a dose similar to that naturally shed by *D. melanogaster* (i.e. 1,000,00 *vs* 5,000) produced different

670 results. The low, natural dose of deposited bacteria failed to elicit avoidance, suggesting *D. melanogaster* repellence is largely due to the production of unidentified molecules when in symbiosis. Symbols indicate means and error-bars standard errors. Statistical tests produced by Wilcoxon signed rank tests; * for p< 0.05; ** for p< 0.01; *** for p< 0.001.




675

Figure 5: effect of *D. melanogaster* larvae and their associated microbiota on the development of *D. suzukii* eggs until adult emergence. Eggs were individually deposited in grape berries where we mimicked natural oviposition by *Drosophila* females and field-like conditions. The greater ratio of *D. melanogaster* to *D. suzukii* egg follows relative infestation intensities observed in the field. The

680 statistical interaction between number of *D. melanogaster* eggs and the presence or absence of their microbe was significant ($F_{2,\ 172}$= 6.46; p= 0.002). Independent contrasts indicate a significant difference between the treatments with and without *D. melanogaster* microbes at high *D. melanogaster* density ($F_{1,\ 174}$= 15.6; p= 0.0001). Moreover, posthoc pairwise Student's t tests showed the treatment with the greatest number of conventional *D. melanogaster* eggs was significantly

685 different from all other treatments. Symbols indicate means; error bars indicate standard-errors; *** for p< 0.001.

690 Table 1: details of the experiments presented in the main text and statistical results.

| **Experiment** question and figure presenting the results | **Method** | **Number of *Ds* females in assay; type of container** | ***Ds* particulars** | ***Dmel* particulars** | **Oviposition substrate** | **Replication and raw statistical results** Reported number of replicates excludes the frequent cases where no *Ds* eggs were deposited during the experiment | **Additional comments** |
|---|---|---|---|---|---|---|---|
| - Do *Ds* females maintain avoidance behaviour over time?<br>- Fig. 2a | We repeatedly assayed the behaviour of the same females over 4 consecutive days renewing the oviposition substrates daily. | - Initially 10 females, some mortality occurred during the 4 days of the experiment<br>- 30cm diameter cylinders | Standard laboratory population | Standard Oregon R laboratory population | - Jellified grape juice<br>- 5cm petri-dishes | We followed 9 cages throughout the 4 days.<br>**Wilcoxon signed rank tests, one-tailed:**<br>Day 1: S= 21; p= 0.008<br>Day 2: S= 20.5; p= 0.006<br>Day 3: S= 6; p= 0.28<br>Day 4: S= 9; p= 0.18 | On days 1 and 3 *Ds* females were given the choice between *Dme*-exposed and control substrates. On day 2 and 4 they were given the choice between *Dmel*- and *Ds*-exposed substrates. |
| - Is avoidance behaviour present in various *Ds* populations?<br>- Fig. 2b | We assayed avoidance behaviour in 4 laboratory populations of different regional origin | - 1 female per assay<br>- 9 cm diameter cylinders | Standard laboratory population and 3 populations founded from individuals captured in China, the USA and Japan | Standard Oregon R laboratory population | - strawberry puree<br>- 2*2 cm cubic receptacles | **Wilcoxon signed rank tests, one-tailed:**<br>China: n= 14, S= 32.5; p= 0.019<br>France: n= 57, S= 557; p< 0.0001<br>Japan: n= 27, S= -3.5; p=0.53<br>USA: n= 16, S= 42; p=0.014 | Details of the tested *Ds* populations are available in the biological material section |
| - Do wild *Ds* females avoid *Dmel* exposed media?<br>- Fig. 2c | We captured wild adult flies and assayed their behaviour in the laboratory. | - 1 female per assay<br>- 9cm diameter cylinders | Laboratory and wild flies | Wild *Dmel* | - strawberry puree<br>- 2*2cm cubic receptacles | **Wilcoxon signed rank tests, one-tailed:**<br>Laboratory: n= 19, S= 74; p= 0.0008<br>Wild 1: n= 12, S= 6.5; p= 0.28<br>Wild 2: n=8, S= -15; p= 0.98 | Wild *Ds* females were trap-captured in two localities near Montpellier, France. Traps usually contained a diversity of fly species, including *Dmel*. *Ds* females were kept in the laboratory for several days before they started laying eggs and could be assayed. |
| - Are male *Dmel* repellent?<br>- Fig. 3a | Oviposition substrates exposed to either males or females *Dmel*. | - 1 female per assay<br>- 9cm diameter cylinders | Standard laboratory population | Standard Oregon R laboratory population | - jellified grape juice<br>- 2*2cm cubic receptacles | **Wilcoxon signed rank tests, one-tailed:**<br>Female *Dmel*: n= 16, S= 54; p= 0.0013<br>Male *Dmel*: n= 21, S= 83.5; p= 0.0007 | When *Ds* oviposition substrates had been exposed to *Dmel* females that oviposited too, eggs from each species could be discriminated thanks to the elongated respiratory tubes that are specific to *Ds*. |
| - Are wild *Dmel* and | We captured wild adult | - 1 female per assay | Standard laboratory | Laboratory and | - jellified grape | **Wilcoxon signed rank** | Details of the tested *Dmel* |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| laboratory *D. simulans* repellent?<br>- Fig. 3b | *Dmel* and tested their repellency. We also tested the repellency of F1 offspring from wild-*Dmel*. A *D. simulans* population laboratory was included in the assay | - 9cm diameter cylinders | population | wild *Dmel*; offspring of- wild *Dmel* flies (i.e. F1); laboratory *D. simulans* population. | juice<br>- 2*2cm cubic receptacles | **tests, one-tailed:**<br>Wild F0: n= 17, S= 57.5; p= 0.0032<br>Wild F1: n= 16, S= 6.5; p= 0.40<br>Laboratory: n=23, S= 62.5; p= 0.027<br>*D. simulans*: n= 19, S= 7; p= 0.40 | populations are available in the biological material section |
| - Is *Dmel* repellence exerted at a distance?<br>- Fig. S2 | We tested whether medium surrounding medium exposed to *Dmel* received less *Ds* eggs than medium surrounding pristine medium. | - 1 female per assay<br>- 20cm cubic netting cages | Standard laboratory population | Standard Oregon R laboratory population | - strawberry puree<br>- 2*2cm cubic receptacles and 12cm square petri dishes | **Wilcoxon signed rank tests, one-tailed:**<br>Exposed area: n= 29, S= 101; p= 0.013<br>Peripheral area: n= 29, S= -14; p= 0.61 | Details of the protocol are described in the supplementary materials |
| - Do axenic *Ds* and *Dmel* flies maintain avoidance and repellence?<br>- Fig. 4a | We produced axenic *Ds* and *Dmel*, which we compared to conventional flies. They were tested in a full-factorial set-up. | - 1 female per assay<br>- 9cm diameter cylinders | Standard laboratory population; conventional and axenic | Standard Oregon R laboratory population; conventional and axenic | - strawberry puree<br>- 2*2cm cubic receptacles | **Wilcoxon signed rank tests, one-tailed:**<br>Conventional *Dmel* and conventional *Ds*: n= 27, S= 133; p= 0.0001<br>Conventional *Dmel* and axenic *Ds*: n= 17, S= 60.5; p= 0.002<br>Axenic *Dmel* and conventional *Ds*: n= 16, S= 19.5; p= 0.11<br>Axenic *Dmel* and axenic *Ds*: n= 16, S= 8.5; p= 0.39 | Details for the production of axenic flies are described in the appropriate section |
| - Test of bacterial candidates possibly involved in *Dmel* repellence<br>- Fig. 4b | We mono-associated axenic *Dmel* flies (i.e. made gnotobiotic flies) with two of their most important bacterial gut reported in the literature, *Lactobacillus brevis* and *Acetobacter pomorum*. We also tested *Dmel* associated with *Escherichia coli*. | - 1 female per assay<br>- 9cm diameter cylinders | Standard laboratory population | Axenic and mono-associated Oregon R flies | - Pieces of bleached strawberries maintained in agar jelly<br>- 2*2cm cubic receptacles | **Wilcoxon signed rank tests, one-tailed:**<br>*L. brevis*: n= 14, S= 40.5; p= 0.0043<br>*A. pomorum*: n= 13, S= 22; p= 0.0745<br>*E. coli*: n=11, S= 10.5; p= 0.18<br>Axenics: n= 17, S= 31; p= 0.071 | Details for the production of axenic and mono-associated flies are described in the appropriate section |
| - Do *Ds* females that bear repellence bacteria still avoid oviposition in sites exposed to *Dmel* associated with the same bacteria?<br>- Fig. 4c | We compared the behaviour of axenic *Ds* females to that of conspecific associated with the same bacterium as in *Dmel* | - 1 female per assay<br>- 9cm diameter cylinders | Axenic and mono-associated with *Lactobacillus brevis* | Oregon R flies mono-associated to *Lactobacillus brevis* | - strawberry puree<br>- 2*2cm cubic receptacles | **Wilcoxon signed rank tests, one-tailed:**<br>Axenics: n= 29, S= 147; p= 0.0003<br>Associated to L. brevis: n= 28, S= 54; p= 0.11 | Details for the production of axenic and mono-associated flies are described in the appropriate section |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| - Are bacteria alone sufficient to elicit *Ds* avoidance?<br>- Fig. 4d | We deposited cells of the bacterium *L. brevis* on oviposition medium, let it rest overnight as for *Dmel* exposure, and tested whether it elicited *Ds* avoidance. This assay was repeated with high and low cell numbers, the latter corresponding to the number of cells retrieved on medium surface after *Dmel* exposure in the standard conditions of our experiments. | - 1 female per assay<br>- 9cm diameter cylinders | Axenics | mono-associated Oregon R flies (i.e. bacteria in *Dmel*) and pure bacteria from liquid culture | - strawberry puree<br>- 2*2cm cubic receptacles | **HIGH bacterial dose (1,000,000):**<br>**Wilcoxon signed rank tests, one-tailed:**<br>*Dmel* exposure: n= 29, S= 147; p= 0.0003<br>Bacteria only: n= 29, S= 103; p= 0.011<br><br>**LOW bacterial dose (5,000):**<br>**Wilcoxon signed rank tests, one-tailed:**<br>*Dmel* exposure: n= 22, S= 82.5; p= 0.002<br>Bacteria only: n= 11, S= 2.5; p= 0.58 | Details for the production of axenic, mono-associated flies and purified bacteria are described in the appropriate section |
| - Does the microbiota of *Dmel* larvae affect their influence on *Ds* development?<br>- Fig. 5 | Details of this experiment are reported in a separate Material and Methods section | | | | | **REML mixed-model:**<br><br>Number Dmel eggs per Ds egg ; $F_{2,169}$= 4.83; p= 0.009<br>Dmel axenic or not; $F_{1,162}$= 0.41; p= 0.52<br>Number of Dmel eggs * axenic or not; $F_{2,172}$= 6.46; p= 0.002<br>Number of emerging Dmel adults; $F_{1,174}$= 7.74; p= 0.006 | Results from the statistical analyses were similar also when neglecting the actual number of *D. melanogaster* individuals that survived and emerged |